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GRANT NUMBER DAMD17-98-1-8078

TITLE: Functional Significance of Transcriptional Regulation by VEGF Receptor Tyrosine Kinases

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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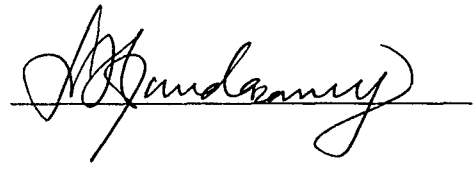

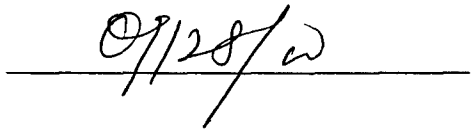

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Subject: Unpublished data in Annual Summary Report for Award Number DAMD17-98-1-8078

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28 July, 1999

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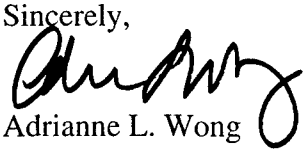

Adrianne L. Wong

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (1 Jul 98 - 30 Jun 99)

4. TITLE AND SUBTITLE
Functional Significance of Transcriptional Regulation by VEGF Receptor Tyrosine Kinases

5. FUNDING NUMBERS
DAMD17-98-1-8078

6. AUTHOR(S)
Adrianne L. Wong

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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13. ABSTRACT *(Maximum 200 words)*

The relationship between angiogenesis and breast cancer tumorigenesis has been well established. VEGF is a leading candidate for a molecular mediator of tumor angiogenesis. VEGF is expressed by tumor cells in vitro and in vivo; blocking VEGF signaling arrests tumor angiogenesis and tumor growth in vivo. Based on these findings, it is clear that understanding the molecular mechanisms that drive the endothelial responses to VEGF will improve our understanding of tumor angiogenesis. Several of the signaling molecules that associate with the activated VEGF receptors influence pathways involved in transcriptional regulation in the nucleus. We hypothesize that the angiogenic effects of VEGF signaling are attributable, at least in part, to the activation of transcriptional regulation of gene expression. Thus, the goal of the study undertaken is to identify genes that are regulated by VEGF RTK activation and to determine the functional significance of these genes in tumor angiogenesis. We have completed two screens, a cDNA Representational Difference Analysis screen and another using the Gene Discovery Array filters. We further explored the role of C3G and CD9 in VEGF-mediated angiogenesis, but ruled them out. We are currently exploring the role of SOCS2, a negative regulatory protein, in the VEGF angiogenic pathway.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Angiogenesis, Vascular Endothelium, Receptor Tyrosine Kinase VEGF, Tie, Transcriptional Activation Angiogenesis Breast Cancer	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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PI - Signature 7/28/99 Date

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Introduction

The relationship between angiogenesis and breast cancer tumorigenesis has been well established. Tumors require angiogenesis to grow and metastasize; the higher the vascular density of a tumor biopsy, the poorer the prognosis. Currently, VEGF is a leading candidate for a molecular mediator of tumor angiogenesis. VEGF is expressed by tumor cells in vitro and in vivo; blocking VEGF signaling arrests tumor angiogenesis and tumor growth in vivo. Based on these findings, it is clear that understanding the molecular mechanisms that drive the endothelial responses to VEGF will improve our understanding of tumor angiogenesis. Several of the signaling molecules that associate with the activated VEGF receptors influence pathways involved in transcriptional regulation in the nucleus. We hypothesize that the angiogenic effects of VEGF signaling are attributable, at least in part, to the activation of transcriptional regulation of gene expression. Thus, the goal of the study undertaken is to identify genes that are regulated by VEGF RTK activation and to determine the functional significance of these genes in tumor angiogenesis. It is hoped that genes regulated by VEGF RTK activation will be useful as biomarkers of tumor angiogenesis or as targets for novel antiangiogeneic therapeutics.

Annual Summary

Specific Aim 1: Establish an in vitro system in which to study VEGF RTKs

We have utilized two different endothelial cell lines in our experiments: ECRF (immortalized HUVEC) and HUVEC (primary Human Umbilical Vein Endothlial Cells). Presence and activation of VEGF and Tie receptors was assayed by Western blotting of immunoprecipitated receptors with antiphosphotyrosine antibodies. As a guideline, the maximal expression of "late" genes induced by FGF occurs between 8 and 18 hours. The two conditions used for screening were ECRF cells at 12 hours post-VEGF stimulation for "late" genes and HUVEC at 6 hours post-stimulation for "early" transcriptionally regulated genes.

Specific Aim 2: Clone genes differentially expressed in the endothelium as a result of VEGF RTK activation

cDNA Representational Difference Analysis (RDA) is a PCR-based subtraction approach to find transcriptionally regulated genes. Briefly, mRNA from VEGF-stimulated and unstimulated populations of ECRF cells was reverse-transcribed into cDNAs, digested into short "amplicons", and put through several rounds of PCR-driven subtraction to find the differences between the two populations. RDA yielded 3 putative upregulated gene products and 5 putative downregulated gene products. Contrary to previously published RDA results, these major bands were in fact a heterogeneous population. These putative regulated gene products were subcloned, and a total of 70 genes were sequenced, BLAST searched, and identified. Northern blotting of RNA isolated under the original conditions utilizing the cloned candidate genes as probes narrowed the field to two interesting candidates: C3G and CD9/MRP-1.

Another differential cloning approach recommended by my committee was to screen the Gene Discovery Array filters (GDA, Genome Systems). GDA filters contain an array of roughly 18,000 human genes from the IMAGE consortium of cDNA ESTs. ^{32}P Probes were made from mRNA from 6 hr VEGF-stimulated or unstimulated HUVEC. Each probe was hybridized to a replicate filter; hybridization was detected on phosphoimager plates and analyzed with the Genome Discovery Software to assess the differences in gene expression between the two conditions. Of the 18,000 ESTs assayed, only 6 ESTs demonstrated an excess of 5 fold expression difference. Northern Blotting was performed using as probes the ESTs that had been identified as potential differential expressors by the GDA screen. Of the 6 potentially regulated ESTs screened, 3 failed to hybridize to Northern blots with a detectable signal, while the other 3 displayed expression levels that were within 1 fold of baseline expression. Possible reasons for the lack of success with this approach include: 1) the timepoint post-VEGF expression was too early, 2) genes relevant to VEGF signaling are activated in a paracrine fashion undetectable by our single cell type system, or 3) the GDA filters simply did not contain targets for VEGF signaling. Therefore, this approach was uninformative.

Specific Aim 3: Characterize the function of genes differentially expressed in the endothelium after stimulation of VEGF RTKs

C3G is a 145 kDa RAS family guanine nucleotide exchange factor for Rap1 and is activated by the expression and binding of Crk adaptor proteins. Crk adaptor proteins are involved in a variety of signaling cascades such as growth factor recptors, integrins, and

cytokines. Interestingly the knockout of B-raf, a member of this signaling pathway, resulted in a vascular phenotype evocative of the Tie2 knockout. While we pulled C3G out of a 12 hour screen, we decided to also investigate its role immediately post VEGF stimulation, when the bulk of cytoplasmic signaling occurs. Western blotting of ECRF cell lysates stimulated with VEGF for 1, 5, 15 minutes or 12 hours demonstrated similar levels of C3G expression compared to unstimulated controls. However, its role might be to differentially associate with other proteins in response to VEGF signaling, so we pursued that line of questioning. In our experiments, C3G did not Co-IP with its upstream partners CrkL or CrkII either with or without VEGF stimulation, despite the abundance of Crk protein in ECRF lysate. In in vitro experiments, there was no association between recombinant GST-VEGF receptor kinase domains and CrkL, CrkII, C3G or Rap1 from ECRF cell lysates. Thus, while C3G is present in the endothelial cell milieu it does not have a role in the VEGF signaling pathway under the conditions addressed.

CD9/MRP-1 is a 24 kDa member of the tetraspanin family of cell surface proteins. It has been demonstrated to play a role in cell migration, matrix adhesion, metastasis, and cellular signaling via association with other tetraspanins and integrins. CD9 is expressed in endothelial cells, vascular smooth muscle cells, platelets, melanoma, and carcinoma. Interestingly, in solid tumors, higher CD9 expression correlates with greater metastatic potential. Thus, CD9 could potentially play a role in VEGF-triggered endothelial cell migration or downstream cellular signaling.

We began with experiments to assess CD9 expression in response to VEGF stimulation. A Northern timecourse performed on total RNA from ECRF cells [fig] revealed a gradual increase of CD9 expression to twofold of baseline by 16 hours post VEGF stimulation. FACS analysis of CD9 cell surface protein expression post-VEGF stimulation revealed a rapid 2 fold increase at 0.5 hr post VEGF treatment. Thus, we hypothesized that VEGF stimulation might trigger the localization of CD9 to the cell surface from a sequestered cytoplasmic location in an event parallel to its gradual transcriptional upregulation.

We assessed the functional role of CD9 protein in VEGF-mediated cell migration via a modified Boyden chamber assay. HUVEC cells were plated on the upper surface of a Transwell chamber (Corning) and we tested their ability to migrate through the membrane towards VEGF in the lower chamber. Here, cell surface CD9 function was disrupted by the addition of Syb-1, a monoclonal anti-CD9 blocking antibody (Dr. C. Boucheix INSERM, France). The addition of Syb-1 resulted in a 50% decrease in VEGF-mediated chemotaxis, as compared to untreated and nonspecific IgG-treated controls. This implicates CD9 in VEGF-mediated cell migration. Thus the working hypthesis was that pretreatment with Syb-1 results in an impediment of VEGF-induced integrin-dependent motility via disruption of tetraspanin/integrin interactions.

To investigate the mechanisms of CD9 cell surface localization from vesicular sequestration, we employed confocal microscopy and timelapse microscopy. Von Willebrand's factor (VWF) was used as a marker for Weibel-Palade (WP) bodies, storage vesicles found in endothelial cells. VEGF stimulation did not result in the fusion of WP bodies with the cell surface as compared to controls. Changes in microfilament organization due to VEGF were tracked with a phalloidin stain as a positive control. Double-staining with CD9 and VWF did not result in significant overlap. Therefore, this suggested that WP bodies do not function as CD9 sequestration vesicles.

Immunocytochemistry experiments viewed by confocal microscopy revealed no obvious differences in total vs cell surface CD9 expression 30 minutes post VEGF stimulation [fig]. This might be explained by a constraint of the system: a two-fold difference in cell surface expression

might not be bright enough to be seen in a single-thickness slice. GFP-tagged versions of CD9 were constructed and transiently transfected into endothelial cells to track the expression and localization of CD9 in real time, which generated similar results. Possible reasons for this observation might be the improper processing or targeting of the GFP tagged protein, a perturbation of the normal cellular responses to VEGF stimulation, or it may well be that the amount of CD9 expression at the cell surface is not quantifiably altered by VEGF stimulation.

In conclusion, CD9 was identified by an RDA screen as a gene transcriptionally regulated by VEGF stimulation of ECRF cells. Its expression increases 2 fold over 16 hours post stimulation by Northern blotting and also 2 fold at 0.5 hr post stimulation by FACS. The addition of a CD9 blocking antibody rendered VEGF–mediated HUVEC migration 50% less efficient. While we could reconcile the somewhat contradictory expression data with the explanation that CD9 was playing an immediate role in migration where VEGF stimulation would drive sequestered stores of CD9 protein to the cell surface, thus necessitating refurbishment by transcriptional activation, we could not find any evidence to support this theory. Thus, we believe that the Syb-1 effect on cell migration is an indirect result of binding CD9 on the cell surface and is not specific to VEGF-mediated cell migration.

Future directions

Upon the completion of two screens for genes transcriptionally regulated by VEGF, I had no positive results. Therefore, it was decided in February 1999 to change direction. My mentor, Dr. Kevin Peters, has left Duke University for a position in industry, though he remains my official thesis advisor. I am continuing my graduate studies in the laboratory of Dr. Chris Kontos, a former fellow trained by Dr. Peters, who is investigating the biology and signaling of endothelial RTKs. My academic committee agreed that pursuing another aspect of VEGF signaling would be preferable to performing another round of screening. While the initial purpose of this proposal was to discover novel genes transcripionally regulated by VEGF signaling, the project that I am currently involved in is focused on earlier events in the signaling cascade, which nontheless may play an important role in VEGF-mediated gene regulation. As the VEGF signaling pathways has by no means been completely elucidated, novel discoveries involving any aspect of this angiogenic process will be exciting additions to the vernacular.

SOCS2 was pulled out of a yeast-2 hybrid screen of a human fetal heart library using VEGFR-2 as a bait in our lab. SOCS-2 is an SH2 domain protein that belongs to the SOCS-box family of cytoplasmic signaling proteins, which function as suppressors of cytokine signaling. We have discovered a novel kinase-dependent association between the endothelial RTKs (VEGFR1, VEGFR2, Tie2) and SOCS2 in the 2-hybrid system. We are currently making a His-tagged SOCS2 protein to test for association with the GST-fused kinase domains of these same RTK proteins in vitro. Studies are underway to map the binding site between these proteins using the specific damins of SOCS2: N-terminal, SH2, and SOCS-box. Furthermore, our collaborator T. Willson at WEHI, Melbourne, AU has agreed to provide us with the transfection constructs for SOCS-1, SOCS-2, SOCS-2 K/R and SOCS-3 for in vivo cellular association and specificity experiments. We are also generating an adenovirus for efficient introduction and overexpression of SOCS-2 into endothelial cells to look at downstream signaling mechanisms. Hopefully, these experiments will prove successful and yield the understanding of a novel aspect of VEGF signaling.

Appendix

Key research accomplishments

- Completed RDA screen for genes transcriptionally regulated by VEGF stimulation of ECRF cells
- Learned how to perform migration assays, confocal and video microscopy
- Investigated role of c3G and CD9 in VEGF-stimulated angiogenesis
- Completed GDA screen

Reportable outcomes

- Offered postdoctoral positions by Dr. Susan Fisher, UCSF (Endothelial RTKs & early implantation) and Dr. Linda Giudice, Stanford (RTKs and endometriosis).

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

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